# VIRUS AND NUCLEIN ACID

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# VIRUS AND NUCLEIN ACID*

by

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Virus research, as a part of experimental biology, has in the last few years made remarkable contributions toward widening our biological knowledge and toward understanding basic biological phenomena. One of the milestones in the progress of this relatively recently branch of science was the experimental proof that the nuclein acid part of a virus is the only real carrier of the genetic makeup through which a new virus can be produced in a living cell.

To point out clearly the meaning of these results we shall briefly define the two terms "virus" and "nuclein acid" and describe their most important characteristics.

Viruses were called "vagrant genes" long before details of their structure and function were known. This catchy definition indeed contains the decisive features of a virus:

its scant spatial dimensions;
that it contains "genetic information";
that it can suffer mutation;
that it is not a complete organism by itself, is without energy metabolism and therefore depends for reproduction on an organism (cell) and its metabolism;
that its minimum chemical makeup consists of nuclein acid and protein.

This very same description would also fit the carriers of hereditary characteristics of any cell. The virus has in addition the characteristic of being "vagrant", that is:

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It can leave the place of its reproduction, namely, the cell, and can penetrate another cell to reproduce again.

The substances we call nuclein acid today were first isolated by Friedrich Miescher while working in the first biochemical laboratory in Tübingen Castle in 1871. A culmination point in the effort of many scientists was reached in 1930 with the realization that there are two different kinds of nuclein acids:

1. "Yeast nuclein acid," first isolated in yeast cells. It cantains "ribose" sugar, hence its name ribose nuclein acid or ribo-nuclein acid (RNS) ["S" for the German "Säure" = acid].

2. "Thymo nuclein acid," first isolated in thymus cells. It contains "desoxy ribose" sugar, hence its name desoxyribo nuclein acid (DNS).

Both nuclein acids have basically the same chemical makeup in a 1:1:1 proportion:

a) Sugar (ribose or desoxyribose);

b) Phosphoric acid;

c) Organic bases (adenin, guanin, cytosin, uracil, thymin)*.

A one-of-each combination of these basic parts is called mono-nucleotid. Their polymerization results in polynucleotids, which is the chemical designation for nuclein acid.

Contrary to organisms, viruses always seem to contain only one kind of nuclein acid, either RNS or DNS. Whenever it appears as if both types of nuclein acids are present in a virus, close examination will reveal that one of them is an impurity with host cell nuclein acid (1). After it became possible to purify viruses sufficiently for a quantitative chemical analysis the additional remarkable fact was revealed: All RNS viruses, plant as well as animal pathogenic, although they very greatly in dimensions and particle weight, seem to contain the same absolute amount of RNS. The relative weight portion of RNS virus particle decreases with increasing weight of the particle (polio virus = circa 25% RNS, particle weight circa 6 million; influenza virus = circa 1% RNS, particle weight circa 200-300 million). Thus, the absolute weight of the RNS portion of all examined virus species containing RNS could be determined and resulted in a constant of a mass equivalent of circa 2 million (2).

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*Each nuclein acid contains four organic bases. RNS contains adenin, guanin, cytosin and uracil; DNS the base thymin instead of uracil.

During the past few years a general uniformity of structure of all virus species has also become recognizable: the nuclein acid of a virus, regardless whether DNS or RNS, as a rule is localized in the center of the particle and surrounded by covering substances, protein in the simplest case, forming a protective layer against outside effects (3). Thus a principle of construction known from the organisms also applies to the viruses: the nucleus or its equivalent, carrier of the genetic substance, is more or less centrally located or at least surrounded by an independent layer.

Of course, such analogy does not justify the conclusion that the nuclein acid constitutes the genetic matter of a virus. It is the purpose of this review to reinforce established findings in order to justify such conclusion beyond any doubt. For didactic reasons the central biological role of virus nuclein acid shall be demonstrated first by indirect, and they by direct proof; the historical sequence of any particular work will be considered only inasmuch as it fits into the pattern of logical development

## A. INDIRECT PROOF OF THE BIOLOGICAL ROLE OF VIRUS NUCLEIN ACIDS

This chapter summarizes all experiments that give some indication of the responsibility of the nuclein acid part of a virus for its infectiousness and for the characteristics of the newly synthesized virus particles; but this chapter does not include direct experiments with isolated nuclein acid.

### 1. Experiments With Plant-Pathogenic Virus Species

In 1948 Markham and colleagues (4) reported that it was possible to separate cleaned turnip yellow mosaic virus into two components by ultra-centrifugalizing. One component sedimented faster, was infectious and consisted of protein and RNS; the other was lighter, proved to be pure protein and was not infectious. However, both components without doubt were definite virus products; they were identical in their electrophoretic mobility and could not be told apart serologically. The only perceptible chemical and physical difference was therefore the presence or absence of RNS in an otherwise completely identical virus protein. In consequence it was assumed that the presence of RNS alone could change a noninfectious into an infectious particle.

As early as 1940 Schramm and Müller (5) determined that the acetification of free amino groups of the protein of tobacco mosaic virus did not affect its infectiousness. Later, Harris and Knight (6) were able to demonstrate that not even splitting off of the remaining threonin rests from the virus protein inactivated a tobacco mosaic virus (TMV).

The first experimental separations of virus protein from RNS were conducted by Bawden and Pirie, later by Schramm et al. (7, 8). Mild alkaline treatment was used by Schramm et al. (9) to break down the virus of TMV into a number of protein subunits and RNS. When the $p_H$ value was lowered, the protein fragments united again to form rod-shaped particles; their physical and serological properties were very similar to the original virus, but they were no longer infectious. This admittedly could have been the result of structural faults incurred during reaggregation of the protein, especially since these particles varied in length and mostly did not again attain the length of an infectious virus particle. But here again the decisive chemical difference was that the reaggregated particles did no longer contain the original RNS.

During the continuation of their work Schramm and colleagues (10) were able to provide further indications to the effect that the presence of RNS, and not the intact protein structure, is responsible for the infectiousness of TMV. By a three-hour treatment of TMV at 10.3 $p_H$ and at a temperature of 0° C it was possible to remove only a part of the protein cover of the virus. The relative RNS portion increased thereby from 6 to 9%, but the sedimentation constant was reduced from 180 s to values 80-160 s. And remarkable was that these viruses, partly stripped of their protein cover, were still infectious. Electron-optical photographs disclosed moreover some details of the TMV structure: RNS apparently stretches as a kind of inner thread through the whole length of the virus particle and is surrounded by protein.

The results of the work described heretofore are once more summarized in Table 1.

Table 1

| Virus Protein | Virus-RNS | Infectiousness |
|---|---|---|
| Amino groups acetylized | Normal | + |
| Threonin split off | Normal | + |
| Reaggregated | Missing | - |
| Normal | Missing | - |
| Partially removed | Normal | + |

These indications already permit the conclusion that the RNS portion of the examined viruses greatly affects the infectiousness of the particles.

## 2. Experiments With Animal-Pathogenic Virus Species

Corresponding examinations with human and animal-pathogenic virus species were more difficult to conduct because it is difficult to produce sufficient amounts of purified virus for chemical examinations. Using radioactive-marked virus, Hoyle (11) employed influenza virus, and Wecker and Schäfer (12) the virus of the classical fowl pest to demonstrate that a ribonucleoprotein originally arrested in the center of the particle is freed and penetrates the cell during the infection of cells. But at least a part of the covering structures, mainly lipoids, seems to remain at the cell membrane. In a subsequent second dissection process the virus RNS is separated from its original protein partner; its free-form existence in the cell interior can then be proved.

New cell growing methods, such as growth of suspension cultures, have lately made possible experiments with polio virus which demonstrate the decisive role virus RNS plays in the infectiousness of particles. But since these experiments were started only after the infectiousness of the corresponding pure RNS preparation was known already, they will not be further discussed here.

Joklik and colleagues (13, 14) presented in a recently published series an excellent experimental example of the genetic function of nuclein acid from animal-pathogenic DNS viruses of the pox group. Berry and Dedrick (15) had already established in 1936 that infectious myxoma virus is produced in rabbits when the animals are infected with a mixture of heat-deactivated myxoma virus and active fibroma virus. Fenner and colleagues (16) reported later that this phenomenon of reactivation is applicable in general for all viruses of the pox group. Jokelik and colleagues (13, 14) then developed the following presentation:

A pox virus, rabbit pox, for example, can be reactivated by another virus of the pox group only if inactivation was caused by change of its cover proteins while its DNS remains completely intact. To be "intact", it seems, the pox DNS must contain a certain portion of protein, located in the so-called central body (see W. Schäfer, Volume 31 of this series). There is reason to believe that this protein holds together approximately 40 molecules, which possibly comprise the total DNS mass of a pox virus.

However, the "reactivating" virus must contain only unchanged cover proteins, while its DNS may also be chemically changed. The results of these experiments shall be briefly summarized in the following.

Virus A: Inactivated by changing of cover proteins (through heating, urea). DNS intact = virus can be reactivated.

Virus B: Inactivated by changing of DNS (nitrogen-lost). Cover proteins intact = reactivating virus.

When A and B together are brought on the choriocallantoismembrane of an incubated chicken egg they will cause the infection of some cells, which will now produce active virus. This virus corresponds exclusively to the type A, the donor of the DNS, of the above example. This proves conclusively that the DNS of a pox virus is the only carrier of genetic information of the particle; and, therefore, the DNS alone determines the properties of the newly produced virus.

### 3. Experiments With Bacteriophages

Considerable knowledge concerning the biological role of virus nuclein acid has been gained with these DNS-containing virus species. Possibly the most important findings in this direction were made by Hershey and Chase (17) in 1952. Widely recognized as valid, this truly classical experiment could indeed be accepted as direct proof of the genetic role of virus nuclein acid. It is here categorized as "indirect proof" only because these authors still worked with the complete phage particle, not with isolated nuclein acid.

Hershey and Chase marked $T_2$ phages in the DNS portion with radioactive phosphorus, in the protein portion with radioactive sulphur. When such particles were brought to join host cells of E. coli it became possible to remove again within a short time the $S^{35}$-containing virus proteins from the bacteria membranes by vigorous mechanical treatment. The $P^{32}$-marked phages DNS, on the other hand, had penetrated the cells. It was even possible to show that it appeared again in the first newly formed bacteriophages. As a consequence it was established that only the DNS and a small fraction of the protein (circa 4%) of a bacteriophage is required to infect a bacteria cell. Thus was proved that the DNS alone contains the entire reproductive capacity of the bacteriophage.

It is difficult to estimate what effect these results will have on subsequent virus and nuclein acid research. But in any case, these results constituted the first convincing experimental proof and

justification of the old theory that virus nuclein acid plays a central role in an infection.

Spizizen (18) and Fraser et al. (19) attempted later to infect cells with phages-DNS alone. And indeed, so-called protoplasts, produced by means of lysozym from E. coli, were successfully infected with osmotically disintegrated phage preparations. Such so-called "shockates" will infect intact bacteria cells only insofar as they still contain a small percentage of original phage particles. Conversely, however, protoplasts cannot be infected with intact bacteriophages, since lack of a complete bacteria membrane prevents absorption by the phages, a pre-requisite for the injection of the DNS into the host cell during a normal infection process. These findings together with those of Hershey and Chase make it highly probable that the partially free-existing phage-DNS of the "shockates" penetrates directly the protoplasts and infects them. But all attempts to isolate further the DNS of the "shockates" and to infect protoplasts with them have thus far ended unsuccessfully. It is conceivable that here, as with the DNS of pox viruses, also a certain protein portion is required in order to tie several DNS molecules, possibly a necessity for an infection, and to enable them to penetrate the cell together. This protein could possibly be the 4% which, in Hershey's experiments, penetrated the cell with the DNS during a normal infection.

Highly convincing as these findings are, for the time being they cannot be accepted as direct proof for the infectiousness of pure phages-DNS.

## B. DIRECT PROOF OF THE BIOLOGICAL ROLE OF VIRUS-NUCLEIN ACIDS

As direct proof shall be listed those findings, which were arrived at with biologically active, but isolated and physic-chemically defined virus-nuclein acids. First shall be described the most important techniques of obtaining biologically active virus-nuclein acid. Since this will mean mainly RNS, the methods described in the following are for obtaining this kind of nuclein acid.

### 1. Techniques For The Extraction Of RNS

a) <u>With Phenol</u>.

The best and at the present probably most widespread technique for isolating high-molecular RNS is the so-called "phenol method",

introduced by Morgan and Partridge (20) and first used on TMV by Schuster and colleagues (21).

A virus suspension in 0.02 molar phosphate buffer, $p_H$ 7.2-7.3, and an equal volume of water-saturated (80%) phenol is vigorously shaken for 8 minutes at +4° C. The watery and the phenol phase can easily be separated again through centrifugalizing. The watery phase is twice more subjected to the same treatment with phenol. The dissolved phenol still remaining in the watery phase is extracted by means of ether, the ether then expelled by nitrogen flow. The resulting product contains nearly all RNS of the original virus preparation, but no evidence of protein. The principle of this method is the preparation of a very fine phenol emulsion in water, with a large total surface of both phases. The denaturated protein dissolves better in phenol than in water, and the RNS better in water than in phenol, which makes possible a complete separation.

b) With Dodecylsulphate.

A second, successfully applied technique is the treatment of virus suspensions with a 1% sodium-dodecylsulphate solution (NSD) ["N" for the German "Natrium" = sodium]. This first separates proteins and RNS (22). The protein can then be removed through precipitation with ammoniumsulphate (23, 24).

c) With Cooking Salt.

A third, less used method is heat denaturation and precipitation of the protein in 0.1 molar cooking salt solution according to Cohen and Stanley (25).

All listed methods have been explained with various variations and improvements, but the principle has not been changed.

The phenol method seems to be the best at the present because of its simplicity and the completeness with which it separates proteins from RNS while affording these macromolecules maximum protection from damage.

## 2. Examples Of Infectious Virus-Nuclein Acid

The following results were derived during the isolation of infectious virus-nuclein acids.

But the examples given in this discourse by far do not include all virus species in which infectious virus-nuclein acids could be isolated. The presentation is limited to such findings as are generally important or at least characteristic for one group of virus species. A more complete listing will be found in a subsequent tabulated summary (see Chapter H).

a) Plant-Pathogenic Virus Species. Tobacco Mosaic Virus (TMV).

It is not only because of historical fidelity that the experiments with this virus are treated in first place. In addition to being the first direct proof for the infectiousness of isolated virus RNS, the order of argumentation of these experiments set also the pattern for all later work with other virus species.

For the sake of a logical presentation the reconstitution experiments by Fraenkel-Conrat and Williams (26) will be treated first. These authors dissociated protein and RNS of the virus through mild alkaline treatment by the method of Schramm (9). Non-degraded virus was cast off in an ultracentrifuge. From the remainder the virus protein was precipitated by means of ammoniumsulphate and twice more subjected to the same process for further purification. The resulting product was then that fraction of the virus proteins which was used in the subsequent experiments. The virus RNS was obtained by the NDS method. Here again the product was further purified by precipitation and reprecipitation with alcohol and then termed as fraction of the virus RNS.

When separated both fractions were not infectious up to the tested maximum density of 1,800 $\gamma$/ml RNS or 52 $\gamma$/ml protein. But when 1% solutions of both fractions were mixed in a proportion of one part RNS to ten parts protein and then incubated for 24 hours at +3° C, infectious nucleoproteins formed while the $p_H$ was 6.0-7.0. The infectiousness of 10-100 $\gamma$/ml of such reconstituted virus preparations was equal to that of 0.1 $\gamma$/ml of the original TMV. The electron-microscope disclosed such products to contain small rods identical in length and shape with TMV, but also such of lesser length. The relative RNS content of the reconstituted nucleoproteins of 5-6% corresponded with that of the virus.

These first experiments were shortly thereafter substantiated by findings of others (27, 28). But interesting as they were, for the time being they only permitted the assumption that both virus RNS as well as virus protein are needed for infectiousness.

Fraenkel-Conrat, however, established clearly by additional experiments the central role of the RNS as the sole carrier of genetic function (24, 29).

The protein and RNS fractions of differing TMV strains were isolated as described above and in various combinations reconstituted as nucleoproteins. In serological tests such mixed products showed that their antigenity always and exclusively corresponds with the one of the TMV strain that had supplied the protein fraction. But the decisive result of these experiments was that when such mixed nucleoproteins were examined as to infectiousness, the symptoms on the host plant were the same as those caused by the "donor strain" of the RNS. Furthermore, the virus progeny isolated from plants thusly infected was now also serologically and altogether in every other respect identical with the strain from which the RNS is the mixed reconstitution products derived.

The conclusion seems compelling that the virus RNS is therefore the sole carrier of the genetical information of the whole virus. But concerning the most outstanding property of a virus, namely its infectiousness, the experiments were still based on guesses. Some of the RNS fractions used in the reconstitution experiments, when placed on the host plant in strong concentrations, had there occasionally caused typical lesions. Since it was improbable that the RNS fraction was contaminated by a remainder of intact TMV it was assumed, that the isolated RNS by itself also may have infectious properties. The first convincing proof of this, however, was submitted around the same time by others.

It is, historically speaking, a rather happy coincidence that these probably most important findings about the biological role of nucleic acids were made at the same location where 85 years before a first description was given of this class of organisms: in Tuebingen.

In 1956 Gierer and Schramm (30, 31) presented in two works their experiments with RNS which was isolated from TMV by the phenol method as described above. When such RNS preparations were applied immediately after their extraction on host plants of nicotiana glutinosa, they caused on them typical TMV lesions. The quantitative proportions are summarized in Table 2.

The RNS preparation thus had circa 2% of the infectiousness of an equal amount (in weight) of TMV. By detailed controls it was proved that this relatively low infectiousness was not the result of remaining impurities of intact TMV. These controls were so thorough and complete that they became standard for all subsequent similar work.

Table 2

| | RNS | | TMV | | |
|---|---|---|---|---|---|
| $p_H$ | γ/ml | Number of Lesions | γ/ml | Number of Lesions | Activity Relation |
| 6,1 | 10 | 153 | 0,09 | 95 | 1,7 |
| 6,1 | 10 | 109 | 0,8 | 445 | 1,2 |
| 7,3 | 1 | 524 | 0,05 | 795 | 2,5 |
| 7,3 | 10 | 815 | 0,27 | 1048 | 1,8 |
| 7,5 | 10 | 998 | 0,27 | 685 | 4,2 |

According to A. Gierer and G. Schramm: To Natural Science Research. 11b, 138 (1956)

(1) Chemical tests disclosed no trace of protein in the infectious RNS preparations. The provable limits for this were at 0.4%. The complement binding test, due to its greater sensitivity, revealed that if any at all, less than 0.02% of native virus protein was contained in the RNS preparations.

(2) Although TMV antibodies completely neutralized intact TMV, in an equal concentration they practically did not influence an RNS preparation of comparable infectiousness.

(3) Small concentrations of ribonuclease (0.1-0.01 γ/ml) still completely eliminated within minutes the infectiousness of RNS preparations, while TMV remained resistant against enzyme concentrations thousandfold higher.

Although the listed controls seem to indicate fully that the infectious principle here debated indeed involves free RNS, they can not yet be taken as definite proof. Particularly in the case of TMV it is quite possible that large parts of the protein cover are removed, as has been explained earlier by Schramm and colleagues (9). Such particles are also RNase-sensitive (32). It is also quite conceivable that chemical and serological methods for proving presence of protein will not fully register when only very small amounts of it remain in the virus RNS. And indeed, it has been questioned at times whether the virus RNS as such can really have infectious properties. The serological tests, however, bear out that each infectious RNS molecule can have only a maximum of 30 amino-acids, while a TMV protein subunit consists of 157 amino-acids (see also later). But Gierer and Schramm followed up with additional controls.

Table 3

| | Infectiousness* | |
|---|---|---|
| | RNS | TMV |
| Control | 998 | 685 |
| With Ribonuclease | 0 | 514 |
| Remainder after ultracentifuge | 242 | 3 |
| Control normal serum | 240 | 157 |
| With Antiserum | 195 | 0 |
| Control | 541 | 311 |
| 48 hours, 20° C | 2 | 130 |
| Sedimentation constant | 31s | 150s |
| Molecular weight | $2 \times 10^6$ | $40 \times 10^6$ |

*The infectiousness is expressed as total number of lesion that were produced on the plant. [According to A. Gierer and G. Schramm: Z. Naturforsch. 11b 138 (1956); 13b, 485 (1958)]

(4) The sedimentation constants of TMV, with 180s, and of the infectious principle in the RNS preparations, with circa 31s, vary greatly. When TMV and RNS were centrifugalized under the condition that particles with a sedimentation constant smaller than 70s were left in the remainder, the infectiousness of the RNS remainder was lowered only very little, while the infectiousness of the TMV remainder disappeared almost completely.

(5) Another difference between TMV and RNS was the relatively great lability of the latter. Diluted RNS solutions lost their infectiousness nearly completely when they were kept for 48 hours at 20° C. Under the same conditions TMV was practically stable.

On the basis of these results the authors concluded, that within the limits of probability the free RNS molecules of TMV constituted indeed the infectious principle in their phenol extracts.

By increased definition of the sedimentation constant and by using the viscosity number as a second, independent parameter Gierer determined the molecular weight of the infectious RNS. It showed to be $2 \times 10^6$ and equals thus the total mass of RNS in a virus particle (33, 34). The major result of these examinations was therefore that each virus particle contains only one molecule of RNS. The congruence of molecular weight of the infectious RNS with the total mass of RNS in a virus particle furthermore represents a finding which by itself — more than anything else — may be taken as proof, that the free virus RNS constitutes indeed the infectious principle of such preparations.

But only a summarization of these findings will underscore this assertion; they will once again appear in tabulated form.

b) Animal and Human-Pathogenic Virus Species

aa) Columbia SK Group: Mengo Viruses. Shortly after Gierer and Schramm published their works, Colber and colleagues (35) succeeded in producing the first infectious RNS preparations from an animal-pathogenic virus, the mengo virus, by means of the phenol method. But in this case the RNS was isolated from virus-infected cells, not from purified virus. Ehrlich-Ascites tumor cells were after 7-day's growth in albino mice infected with the virus through intraperitoneal injection and were ready after another 60-68 hours. After repeated washing, the cells were quick-frozen and ground to a fine powder in a mortar. The powder was then homogenized with citrate-buffered cooking salt solution in a Waring blendor. The remainder of the 10% suspension served as base material for the phenol extraction, which was conducted according to Gierer and Schramm in three cycles at circa +4° C.

It was possible to prove the infectiousness of the resulting nuclein acid fractions by intracerebral injection in mice; it was circa 0.1% of the infectiousness of the original homogenate remainder.

In order to prove that infectiousness was not caused by rest virus the authors performed substantially the same controls as described in the preceeding chapter: biuretreaction indicated that protein was not present. Paper-chromatographical examination of the alkaline hydrolyzed RNS also revealed no presence of amino acids in the preparations.

The infectiousness of the RNS fractions was sensitive to RNase, the virus was resistant. Six hours of incubation at 37° C destroyed the biological activity of the RN, but not the one of the virus.

Virus and infectious principle of the RNS preparations furthermore showed varying sedimentation speeds in the ultra centrifuge. But no efforts were made to determine this sedimentation constant.

An additional difference between virus and infectious RNS was that the latter could be precipitated in a 1-molar NaCl solution. The precipitation occurred after 12-16 hours. The precipitants cast off in the centrifuge were again soluble with the original volume of physiological cocking salt solution and had also the original infectiousness, while the infectiousness of virus solutions was considerably lower after the same treatment.

Results of these experiments are shown again in Table 4.

Table 4. Differences between mengo Virus and RNS fraction gained therefrom

| | Virus $LD_{50}$ | RNS $LD_{50}$ |
|---|---|---|
| Control | 6,5 | 2,6 |
| After RNase | 6,6 | 0 |
| 6 hours, 37° C | 6,4 | 0 |
| U after Ultracentrifuge | 3,5 | 2,2 |
| Control | 7,0 | 3,3 |
| Precisely 1 M NaCl | 3,5 | 3,5 |

According to J. S. Colter, H. H. Bird and R. A. Brown: Nature (London) 179, 859 (1957)

The brains of mice successfully infected with the RNS preparations contained normal mengo virus which could be neutralized with specific antiserum.

Mice Encephalomyocarditis Virus. As in most experiments to extract infectious RNS from animal-pathogenic viruses, in this case, too, infected tissue, namely mice-ascites tumor cells, was used as a starting base. Phenol was used for the extraction (36).

The RNS fractions produced plaques in tissue cultures, but this testing method did not offer a good possibility for quantitative evaluation. The best proof was to infect growing ascites tumor cells with the RNS and then to inject them into the mice, which then showed typical symptoms.

Physical-chemically the RNS preparations showed in ultra-violet light the absorption spectrum typical for nuclein acid. All RNS fractions contained amounts of 1-7% protein. Contrary to the virus, the RNS could be precipitated with alcohol or a 1-molar cooking salt solution without detrimental effect on the infectiousness. The RNase sensibility [Translator's Note: Does author mean "sensitivity"?] of the preparations was also proved.

It is of special interest that here too, as with EEE and WEE (see paragraph bb) below), the extracted RNS seems not to have derived from virus particles. The authors found no direct correlation between the RNS yield and the contents of infectious virus. Whether the initial base contained $2.8 \times 10^7$ or $2.2 \times 10^8$ infectious virus particles/ml, the RNS titer remained the same with $3\text{-}5 \times 10^3$. It was furthermore possible to show that virus particles and the components, from which

the infectious RNS is extracted, were separable in the ultra-centrifuge, as is shown in Table 5.

Table 5

| Base Material | Virus $LD_{50}$/ml | RNS $LD_{50}$/ml |
|---|---|---|
| Tissue culture liquid | $5{,}2 \times 10^8$ | $6{,}3 \times 10^3$ |
| Remainder after 1 hour, 100000 g | $2{,}2 \times 10^5$ | $3{,}0 \times 10^3$ |
| Sediment after 1 hour, 1000000 g | $1{,}2 \times 10^8$ | $1{,}6 \times 10^2$ |
| Sediment washed and concentrated | $3{,}0 \times 10^9$ | 0 |

According to J. Huppert and F. K. Sanders: Nature (London) 182, 515 (1958).

This indicates that the infectious RNS most likely is not derived from the infectious virus. But contrary to EEE and WEE, the extractable component as well as the virus proper cannot be very sensitive to RNase.

Thus far nothing has been published about experiments to isolate infectious RNS directly in virus particles.

Mice Encephalomyelitis Virus (ME). The brains of infected mice served as base material. The RNS was extracted by the usual phenol method or with phenol at increased temperature, as has been described for EEE or WEE. Infection tests were conducted by intracerebral injection of mice. Virus particles were the only source of infectious RNS in these experiments by Franklin and colleagues (37). It was also possible to isolate infectious RNS from virus preparations which had been extensively purified by fluorcarbon (38). Such RNS showed the usual property differences when compared with virus.

While corroborating and elaborating the findings gained from EEE (see 66) it was possible to show that the cellular DNS present in extracts from infected tissue exercises an inhibitive influence on the infectious RNS. The absolute RNS titer rose when the DNS was destroyed by DNase or when the RNS was separated from the DNS through precipitation with 1-molar cooking salt solution. Similarly, the dosis-effect curve for the RNS now came closer to the theoretically demanded prediction curve, which was previously not the case.

According to a subsequent announcement by Haussen and Schäfer (39), a first-stage RNS can evidently also be obtained in the case of ME virus

from cells infected with it. The RNS will appear one to two hours before commencement of the virus reproduction proper.

The same work contains the remark that the virus here designated as mice encephalomyelitis virus has been found to be close to the viruses of the Columbia SK group as a result of newer examinations.

bb) Viruses of the Group Encephalitis A: Virus of the American Horse-Encephalomyelitis (Type East and West) (EEE and WEE). Different than the virus species described thus far, the particles here are relatively large (45 mμ) and furthermore have the very high lipoid content of 54% (40).

These peculiarities require certain variations in the technique of extraction of infectious RNS. Wecker and Schäfer followed somewhat the experiments of Colter (35) in obtaining the first infectious RNS of EEE virus from virus-infected mice brains (41). The frozen mice brains were immediately homogenized while the phenol was present. The watery phase separated in the centrifuge was then treated in three additional phases with phenol at low temperatures. Such preparations caused typical infections after intracerebral injection into mice. With them, it was possible to produce plaques on tissue cultures of chicken fibroblasts. But the plaque test could not be quantitatively evaluated because of its irregularity with RNS preparations (see also Polio). The infectious RNS preparations had the usual sensitivity toward RNase and prolonged incubation at 37° C. The RNS differed from intact virus also in that it could be precipitated with alcohol without loss of infectiousness, while such treatment completely inactivated intact virus.

Infected chicken embryos served as base material for additional experiments. The infectiousness tests were also performed with incubated chicken eggs.

During the experiments with infected mice brains as well as during all subsequent experiments it was found that only when phenol was already present during the homogenization of the tissue, it was possible to obtain an optimum yield of infectious RNS from infected chicken embryos. This was contrary to the findings made with TMV and mengo virus. Furthermore, it was shown (see Table 6) that a short RNase treatment of the tissue homogenates prior to the phenol extraction completely inactivated the extractable RNS. Colter et al. (35) and later Franklin et al. (37) found that Rnase did not influence the extractable RNS when the enzyme was added prior to the phenol treatment. The conditions of EEE virus and mice encephalomyelitis virus are compiled in Table 6 for the sake of comparison.

Table 6

| Technique | EEE* $LD_{50}$/ml | MEV** $LD_{50}$/ml |
|---|---|---|
| Homogenized with Phenol + 3 times Phenol | 3,2 | 3,0 |
| Homogenized without Phenol + 3 times Phenol | 2,02 | 3,0 |
| Homogenized without Phenol 1- min RNase 1 γ/ml + 3 times Phenol | 0 | 4,0 |

*According to E. Wecker: IV. International Congress to Biochemistry, Vienna, 1958.
**According to R. Franklin: Virology 7,220 (1958).

From these results it was concluded, that the infectious RNS which can be isolated in EEE-infected tissue is already present in an Rnase-sensitive form prior to the phenol treatment. But since the EEE virus, as all other virus species, is completely resistant to this enzyme, it was assumed that the extractable infectious RNS did not derive from the virus particles as such. And indeed, it could be shown that a thoroughly purified WEE virus preparation with an infectiousness more than hundred-fold greater than that of tissue homogenates yielded no more infectious RNS after three extraction cycles with phenol at low temperatures.

The RNS derived from infected tissue was therefore most likely virus nuclein acid not contained in virus particles or not yet incorporated in them (42).

The extraction of infectious RNS from virus particles proper became later possible through increased temperature during the phenol treatment (43). In the case of EEE and WEE it was assumed that lipoids form a cover structure around virus particles which could not be sufficiently dissolved with phenol at low temperatures. The fact that with rising temperature the RNS yield of a virus preparation increases in volume and infectiousness, as is shown in Table 7, added credibility to this view. The corresponding results with TMV are shown for comparison.

The NS fractions from infected tissue showed three main gradients in the ultra-centrifuge. They had the following sedimentation constants:

Gradient A (RNS) 30-32 s.
Gradient B (RNS) 19 s.
Gradient C (DNS) circa 12 s.

Table 7

| Temperature in °C | WEE Total RNS Extracted γ/ml | Infectiousness $Id_{50}$/ml | TMV Total RNS Extracted γ/ml | Infectiousness Lesions |
|---|---|---|---|---|
| 4 | 0 | 0 | 0,93 | 94 |
| 40 | 72 | 2,35 | 1,58 | 112 |
| 50 | 710 | 3,24 | - - | - - |
| 60 | - - | - - | 1,33 | 87 |

According to E. Wecker: Virology 7,241 (1959).

The molecular weight determination resulted in a value of 2 X $10^6$ for the RNS of the gradient A (44). Examinations of the sedimentation characteristics of the infectious principle indicated, that it decreased together with the gradient A. From this it was concluded that the infectious RNS molecule in the nuclein acid fractions of EEE-infected tissue has a molecular weight of approximately 2 X $10^6$. They are, therefore, in all tested chemical and physical aspects identical with the infectious RNS of TMV (citation from 45). This justified also the conclusion that the infectious component, as is the case with TMV, is most likely made up of free RNS molecule. The important differences between virus and infectious RNS are given in Table 8.

Table 8

| | Virus $LD_{50}$/ml | RNS $LD_{50}$/ml |
|---|---|---|
| Control | 8,1 | 3,0 |
| After Alcohol Precipitation | 0 | 3,5 |
| With 25 /ml RNase 30m in 37°C | 7,7 | 0 |
| 4 hours at 37°C | 6,7 | 0 |
| Control | 6,1 | 3,8 |
| With Normal 1:100 | 6,2 | 0 |
| Molecular Weight | circa 50 X $10^6$ | circa 2 X $10^6$ |

According to E. Wecker: On Natural Science Research. 14b, 370 (1950); 15b, 71 (1960).

cc) Viruses of the Group Encephalitis B: Murray Valley Encephalitis Virus (MVE). An infectious RNS fraction was extracted with phenol from the infected brains of newborn mice (46, 47). The infectiousness of the extracts was RNase-sensitive and was inactivated with 10%-normal serum from rabbits.

Noteworthy are the findings of Ada and Anderson that material with identical characteristics can be obtained by treating unpurified MVE virus with 1% desoxycholate at 20° C. Whether the infectious principles are completely identical after phenol or desoxycholate treatment has not been ascertained, but it is probable. Thus, in this case, the virus particle would release its infectious RNS after removal of the lipoid cover by means of desoxycholate; unless it is proved that from MVE, too, an infectious RNS can be isolated, that is, from some other, easier extractable material, not only from the virus particles proper.

dd) Small Virus Species: Poliomyelitis Virus. Several groups of collaborators have worked on extraction of infectious RNS from polio virus (48, 49, 50, 51, 52).

Colter and colleagues (48) isolated the RNS in the ZNS of hamsters infected with type 2 polio virus. Again, the same technique as for mengo virus was employed. The infectiousness of the preparations was proved by intracerebral injection into mice.

Alexander and colleagues (49, 50, 51) used in their experiments concentrated and partly purified type 1 polio virus. The RNS was again extracted by the phenol method as described by Gierer and Schramm for TMV. It was possible to produce plaques on hela-cell cultures with this RNS. But at first this plaque test was unsuitable for a quantitative evaluation of the infectiousness since, with undiluted RNS preparations, confluent cytopathogenic lesions appeared; and with diluted RNS, no significant linear proportion between dilution factor and plaque value could be observed. In qualitative experiments the authors noted, that the infectiousness of their RNS preparations was destroyed by 100 $\gamma$/ml RNase, while an identical concentration of DNase had no effect. Proteolytical ferments, such as papain and chymotrypsin, also did not destroy the infectiousness. A dilution of 1:10 of monkey serum completely inactivated RNS, regardless whether a specific antiserum or a normal serum was used. The authors assume that this "unspecific" effect can be attributed to the presence of RNase in the serum. And indeed, the plaque formation with RNS was not prevented by $\gamma$-globules of antiserums. But the same concentrations still completely neutralized an equivalent amount of polio virus.

Together, the findings of the two work groups let it appear very probable that the infectious principle in RNS preparations from polio virus is also made up of free RNS molecules. But unfortunately are these experiments lacking in thorough physical and biophysical examinations to give a complete picture.

Schaffer and Mattern (52) later isolated an infectious RNS from high-grade purified polio virus. It was possible to do this with all three techniques (see above). The infectious component was left in the remainder when the RNS fractions were centrifugalized at 100,000 X g. It could be precipitated with alcohol and was sensitive to RNase. The infectious RNS furthermore had the absorption in ultraviolet light which is characteristic for nuclein acids.

Virus of the Foot and Mouth Disease (MKS). This is one of the smallest and simplest animal-pathogenic viruses. Several groups of collaborators were able to obtain infectious RNS preparations by means of the phenol method (53, 54, 55, 56).

As a first step the RNS was isolated from infected tissue and its infectiousness was demonstrated by injection into mice. While the titer of virus was equally high in intraperitoneal and intracerebral application, good results were obtained with RNS only in case of intracerebral injection (54).

RNase and incubation at 37° C destroyed the infectiousness of the RNS preparations. Equally, inactivation was caused with normal serums diluted up to 1:10,000. With an immune-globuline fraction diluted to 1:100, however, virus could still be neutralized, while RNS was unaffected.

Strohmeyer and Mussgay (57) also determined the molecular weight of the infectious component in RNS fractions, using as a base the relation between sedimentation constant and molecular weight which Gierer had given for the RNS of TMV (33, 34).

The sedimentation constant was examined through centrifugalizing in a $D_2O$-$H_2O$ density gradient.

The obtained value of 37 s is somewhat higher than those derived from TMV and EEE (33, 43). From this the molecular weight of the infectious RNS was computed as being $3.1 \times 10^6$, and it is consequently — by circa 30% — again greater. The difference lies perhaps in the various techniques which were used in determining the sedimentation constant.

Another important observation was made by Brown and colleagues (55). When they examined infected cells and the remainder of infected

cells for virus contents and extractable RNS it was found that invariably more infectious RNS could be extracted from the cells, regardless whether the virus titer was high or low. It seems therefore that the cells of MKS also have a second source of extractable virus RNS which is not the virus itself.

On the strength of experiences with the infectious RNS of the MKS virus a surprisingly simple and logical explanation was finally found for the earlier-discovered phenomenon that this virus loses its infectiousness already in a slightly sour milieu, namely at $p_H$ values between 6.0-6.5 (58). Mussgay was able to prove that at such $p_H$ the virus disintegrates in so-called S-antigen-subunits, which probably constitute the virus protein. The RNS is thereby released. If the experiments are performed with unpurified virus preparations, then the slight impurity with RNase suffices to inactivate the nuclein acid immediately. But if purified virus is used, an infectious component is obtained which will withstand even lower $p_H$ values and which corresponds in all examined characteristics with virus RNS (59).

ee) Myxo Viruses: Influenza Virus. The results regarding an infectious RNS from this virus species are, to say it prudently, still much debated. Positive results have been reported by only two work groups, while most likely several other groups did not report their negative findings.

R. Portocala and colleagues (60, 61, 62) reported successful isolation of an RNS from influenza-A virus, which had been defattened with ether, by means of phenol at low temperatures; this RNS simulated the production of "homologous" virus in the Allantois cavity of deembryonized chicken eggs. But in two additional works (61, 62) it was found that the virus produced by RNS-infected cells reacted antigenwise slightly different than the virus from which the RNS was derived. In addition, filamentous formations appeared after a second passage in the fertilized chicken egg; these formations were absent in the original virus. The infectiousness of the RNS extracts was sensitive to RNase, incubation at 37° C, or prolonged standing at room temperature.

Maassab (63) reported shortly thereafter the isolation of an infectious RNS preparation from chorioallantois membranes of chicken eggs which had been infected with the Asiatic strain of influenza-A virus. This again was accomplished with phenol at low temperatures. But their infectiousness was proved only by producing a cytopathogenic effect in tissue cultures of embryonic chicken kidney cells, not by injection into the chorioallantois cavity of the incubated chicken egg. This seems all the more surprising since Wecker and Schäfer had found the incubated chicken egg an entirely suitable test system for infectious

RNS of EEE and WEE (41). But otherwise Maassab's infectious agent responded the very same as infectious RNS in its RNase sensitivity, sedimentation characteristics, and precipitation with a 1-molar cooking salt solution. It is therefore possible that Maassab isolated some other RNS, perhaps from a virus latently present in the base material. The progeny of the RNS-infected chicken kidney cells was unfortunately not thoroughly characterized; but it can be observed that this progeny, although supposedly consisting again of intact virus, was not infectious for the incubated chicken egg. If in all previously described cases the virus RNS, as the true "carrier of genetical information", stimulated the production of normal and corresponding virus in cells infected by it, then such definition cannot be applied to the influenza RNS obtained by Maassab.

Only Ada and colleagues (64) reported about their negative attempts to isolate infectious influenza RNS. Included in their experiments was purified virus and virus-infected tissue as well as various influenza-A and B strains. They were also unsuccessful with another representative of the myxo viruses: the Newcastle disease virus. They were even unable to prove partial biological activity of their RNS preparations. While recombinations can be produced with intact virus, this was not possible when RNS from one strain and active virus from another was used. Burnet and his collaborators came therefore to the conclusion that of the examined RNS preparations from influenza virus, none had any kind of biological activity.

In a later chapter of this review will be listed several theoretical deliberations pointing out the seeming improbability of obtaining from this virus species an RNS which is infectious in the usual sense.

ff) Tumor Viruses: Polyoma Virus. This is the first DNS virus from which an infectious nuclein acid could be isolated. It should also be mentioned at this point that the very first example of direct biological activity of isolated nuclein acid was also given with DNS. This is a phenomenon called transformation, in which bacteria in the DNS of a donor strain transfers a certain biological characteristic to a receptive strain which does not possess this characteristic (65). But it seems that in this case only part of the distance of a DNS molecule is required (66). Dimayorca and colleagues (67) reported the first successful isolation of an infectious virus DNS, namely that of the polyoma virus. This work is also of special interest because it is at the same time the first instance of infectious nuclein acid derived from a tumor virus (68).

As base material served virus-infected embryonic mice cells in tissue culture. The DNS was extracted by the phenol method, as was the case with TMV. As a control, DNS was extracted from noninfected

identical tissue cultures by the phenol method as modified by Kirby (69).

The DNS preparations then were planted on tissue-culture cells of embryonic mice tissue. There the virus DNS produced regularly the cytopathogenic effect typical for the virus; the controls remained negative. From the remainder of these virus-DNS-infected cells it was possible to obtain normal polyoma virus, which produced in mice the characteristic tumors. The infectious nuclein acid preparations were sensitive to DNase, insensitive to RNase.

These results have since been repeated and verified by Dmochowski and colleagues (70).

Shope Papilloma Virus. As is often the case, the first success with infectious DNS had another one in its wake: Shortly after an infectious nuclein acid fraction had been successfully isolated from polyoma virus, Ito (71) reported his positive results with a different DNS-containing tumor virus: the Shope papilloma virus.

A nuclein acid preparation was obtained from papillomatious warts of cottontail rabbits by means of phenol at low (+4° C) and high (+50° C) temperatures. It caused on the skin of domesticated rabbits after intracutaneous or application on the lightly scratched skin within 2 to 6 weeks typical papillomas, which even microscopically were fully identical with those produced by intact Shope virus.

The NS fractions were sensitive to DNase (2 γ/ml), but not to RNase. In contrast, heat-inactivated DNase was without effect, so that an unspecific mechanism could be largely barred. Concentrations of antiserum which still fully neutralized intact virus did not lower the infectiousness of the NS fractions.

In continuation of his studies Ito was able to show that an infectious DNS can also be extracted from partially cleaned Shope papilloma virus with cold and hot phenol (72). But that not only ripe and infectious virus particles are the source of infectious DNS was shown by one extracted from papillomas; the infectious virus content of this DNS practically equalled zero. This result is of particular importance because it permits the assumption, that in certain virus tumors the original releasing agent, namely the intact virus, may have "disappeared", although its genetical material quite definitely is being reproduced in the tumor cells and most likely is even essential for the formation of the tumor.

## C. INFECTIOUS RNS AND HOST CELL

The low relative infectiousness of virus RNS, which generally lies between 2% and 0.001% (see H, Tabular Summarization, p. 33), frequently gave reason to doubt the validity of the obtained results. This chapter shall therefore treat this important point.

As can be seen in the preceding chapter, the infectious RNS preparations were as a rule tested in the same host organisms as the corresponding virus, whether they were certain organ cells of plant and animal or corresponding tissue culture cells.

First arises the question how a molecule of the dimensions of an infectious RNS strain is to penetrate a living cell without being equipped with a specific absorption and penetration mechanism. In the case of the intace virus they are regularly found in the outer cover of the elementary particle, regardless whether they are certain enzymes (bacteriophages, myxoviruses) or just specific characteristics of the cover structure (for example, protein cover of the polio virus).

With TMV, from which the first infectious RNS was isolated, this problem is nonexistent. And the virus itself moves quite naturally into the interior of the cell: through an insect bite or, in the laboratory, through fine openings caused by rubbing off of leaf hair.

It is quite different with animal cells. Here it must be assumed that the infectious NS molecules really enter through the intact cell membrane. This apparently seems to be a cellular activity. Therefore, it is only required of the nuclein acid molecules that they have a total electrical charge pattern permitting them to move sufficiently close to the cell membrane. Then the penetration can take place, either by way of a phagocytosis or through "functional openings" in the membrane. But nothing definite is known about this.

However, it makes sense that an infectious nuclein acid molecule, due to the absence of special mechanisms, has probably a much smaller chance of penetrating than a virus particle. An added disadvantage of the isolated genetical virus material is its great lability. Thus, if such a nuclein acid molecule is not fairly quickly (within circa 15 minutes) absorbed and received by the cell, its inactivation is almost certain.

Together, these two factors — small penetration probability and very great lability — may well be the reason for the lower infectiousness of nuclein acid preparations than that of corresponding virus preparations.

Another reason may be that possibly not all nuclein acid molecules originally present in the virus particles survive intact the process of extraction. As the examinations of Gierer (45) have shown, a single break somewhere in the chain of infectious RNS from TMV is sufficient to destroy the biological activity of the molecule. But in the case of TMV Fraenkel-Conrat (86) also proved, that an RNS preparation with the relatively low infectiousness of 0.5% could assume a relative infectiousness up to 80% after reconstitution of virus particles of such RNS and from isolated virus protein. This seems to prove that at least 80% of the isolated RNS had been present in infectious form, although the direct test indicated only circa 0.5% of this. In the case of WEE virus, however, it must be assumed that indeed only a negligible part of the RNS isolated from the virus particles is still physically intact. The relative infectiousness of such preparations is approximately 1/100,000% (43)! The extraction of the RNS from the virus particles is of course especially difficult in this case and altogether possible only by drastical methods. Although examination results are still lacking, it seems reasonable to assume, that the great differences in the quoted relative infectiousness of virus nuclein acids is partly due to the varying ease with which they can be obtained. It makes sense that the more molecules will remain intact the easier and more gentle the isolation technique is.

But of even greater importance for the relative infectiousness of nuclein acid preparations are the ways and means of testing it. The optimal test method for a virus is not always the best for the corresponding nuclein acid. MKS virus, for example, shows a slightly higher titer at intraperitoneal than at intramuscular application. Conversely, the infectiousness of RNS from this virus can be proved only by intramuscular or intracerebral injection (54). There are several other similar examples (42, 46, 47, 51).

During the evaluation of relative infectiousness of nuclein acids it may very well be that the test method as such causes differences of three powers after ten. But conversely it is also possible to infect non-host cells for a virus with the corresponding RNS. Holland and colleagues (84, 87) were able to show that it was possible to infect with infectious virus preparations from poliomyelitis virus Type 1 even non-primate cells of animals, such as rabbits, hamsters and guinea pigs. For none of these cells or animals is the intact virus infectious. From these significant experiments can be concluded that at least a part of the so-called host specificity of a virus refers to its surface characteristic, in this case to its cover proteins. Their absence, for penetration purpose usually a disadvantage of the RNS as compared with virus, in this case turns out to be a blessing. Another important finding is that apparently many more various cells than was expected possess in principle the same biochemical ability to synthesize the same kind of RNS and protein, namely complete virus. The

same results could also be obtained with other enteroviruses, Echo-8 virus, and Coxsackie A 9 and B 1 (84). The outstanding importance of the test system in quoting the relative infectiousness of virus nuclein acids here once more becomes very clear: In these cases the nuclein acid is even more infectious than the virus!

When testing infectious RNS preparations from animal-pathogenic virus species in an animal, it seems that the intracerebral injection gives generally the highest titer. It is assumed that the relatively low RNase activity of this organ system is one of the reasons for this (88).

As already mentioned in the preceding chapter, the results with the plaque test were at first considerably worse. But meanwhile this has changed. Alexander et al. (50, 51) were the first to observe that not only did the absolute plaque count increase with RNS, but that also the theoretically demanded converse proportionality between plaque count and dilution factor was obtained when the RNS dilution was produced in hypertonic cooking salt solution. These findings were verified by Holland et al. (84). Ellem and Colter were the first to point out in their work the possible effect mechanism of this method. They found out that even better results could be obtained with mengo RNS and the L strain of mice fibroblasts when the RNS was placed on the cells in a high-molar Sucrose solution. Optimal concentrations resulted with NaCl as well as with sucrose, but when the optimum was passed, the plaque count was lowered again. There seems to be some kind of compromise between the highest possible molarity of the solution to the advantage of the RNS, which still makes barely possible the survival of the cells under such unphysiological hypertonic conditions. In most cases such treatment will cause the disintegration of the cell tissue, which of course makes regular plaque formation impossible. The "infective center" technique employed by Ellem and Colter permitted a circumvention of this condition. The suspended cells with the RNS are thereby infected in a hypertonic solution. The osmotic pressure is then normalized again by suspending the infected cells in physiological media, and the cells are subsequently brought to settle on normal cell sod. After covering this with a layer of agar nutrient, a plaque developed on every point of the indicator cell sod on which an infected cell had settled.

These experiments made it clear that the effect mechanism of high-molar solutions in this case is to be found at the cell, not on the RNS as such. Originally it had been assumed that the 0.8-molar NaCl would "stabilize" the RNS, perhaps by changing its physical structure in solution, since it is known that the RNS can be already precipitated with 1-molar cooking salt, although at lower temperatures. But since sucrose in such solutions as have come to be used certainly does not have such effect it is now assumed that through

the osmotic pressure of the extracellular medium the intracellular electrolyt-concentration correspondingly rises. And indeed, the optimal concentrations for NaCl as well as for sucrose correspond to the same osmotical pressure of the solution. Thus, through an increase of the intracellular ion strength a retardation of cellular RNase activity could be effected, as it has been demonstrated in-vitro. But some other mechanism may be just as workable. As has been reported by the authors, the infectiousness of mengo RNS can be destroyed through all proteins free of RNase. Similar observations had already been made by Wecker (42). No inactivation occurred at high ion concentration. It is therefore possible that hypertonic intracellular conditions prevent this kind of unspecific inactivation of RNS through cellular protein.

Within the scope of this chapter it is of interest that the absorption to -- and penetration into -- cells of intact virus under the same hypertonic conditions becomes radically poorer. Let it also be said that when comparint the infectiousness of virus and corresponding RNS preparations, allowance must be made for many factors which are only partially known. And such comparison makes of course only sense if the infectious RNS is really derived from the infectious virus particle and not, as in some of the above-described cases, exclusively or additionally from some other virus-specific material in the infected cells.

## D. THE PRELIMINARY-STAGE RNS

The observation that infectious RNS cannot only be extracted from ripe virus particles but also from an intracellular virus-specific material was the reason for an examination of the timely connection between the reproduction of this material and the virus itself. Such experiments were conducted with cells which had been infected with mice encephalomyocarditis virus (89), the virus of the foot and mouth disease (55), the horse encephalomyelitis virus (90), the tobacco mosaic virus (91, 92), and the ME virus (89). In all cases it was found that an infectious RNS appears already in the virus-infected cells prior to the provable synthesis of the new virus particles. This kind of RNS can therefore rightly be called a "preliminary nuclein acid" since it obviously constitutes a significant pre-stage in the reproduction of virus.

In the case of TMV, it was a little more difficult to prove a preliminary-stage RNS because the usual phenol treatment also affects the RNS already present in the virus particle. Thus Engler and Schramm (91, 92) were able to determine it only indirectly. When they

homogenized the infected leaves and incubated them subsequently for one hour at 37° C, the pre-stage RNS was destroyed by the cell-contained RNase, and only the RNS present and protected in the virus could be extracted. But when the infected leaves were already homogenized in the presence of phenol, the phenol immediately destroyed the cellular RNase, and the sensitive pre-stage RNS as well as the RNS already incorporated could be obtained. The difference in titer between the two preparations was taken as a measure for the pre-stage RNS. By means of this technique it was proved that an infectious RNS appears already 10 hours prior to the provable virus reproduction. At the moment when the virus formation begins, the "free" pre-stage RNS decreases. This was interpreted to mean that the two syntheses take place independently of each other and that the protein synthesis occurs faster than the RNS synthesis. Such conclusion was imminent since it is known that a TMV consists of only one RNS molecule but circa 2,200 protein molecules.

Sanders (89) was able to extract an infectious RNS from ascites tumor cells already 15 minutes after infection with encephalomyocarditis virus (EMC). In the following 5 hours it multiplied 100-1,000 fold. The RNS reached its maximum titer at approximately the time the formation of the first new virus particle began. The virus synthesis as such reached its maximum circa 7-8 hours p.i. In the case of EMC it was possible to measure the pre-stage RNS directly because due to the applied technique the completed virus particle apparently did not release its RNS (36).

Sanders assumes that the first immediately provable RNS derives from those infected viruses that were changed into an extractable form during the virus eclipse. The new-synthesis of RNS as such is said to begin only 1 hour p.i. As with TMV, here too, the titer of the pre-stage RNS decreases from the time the new virus appears. This may mean that now the so-far extractable pre-stage becomes inextractable because of incorporation into the virus. But it is also conceivable that the RNS, not yet incorporated and therefore not stabilized, is being progressively destroyed, since at the same time the first cytopathogenic effects become visible on the cells. Contradicting such interpretation, however, are Sanders' experiments with inhibitors (89). Euflavin retards the formation of pre-stage RNS. When the inhibitor is added 4 hours p.i., e.g., only after the RNS synthesis has reached its maximum, there still will appear no infectious virus. Although the cells apparently have the usual cytopathogenic effect, the RNS titer remains longer than when virus synthesis is possible.

The circumstances were different with the inhibitor 5-fluor uracil. This pyrimidinanalogon is incorporated into nuclein acid. The dosis-effect relations in regard to retardation of RNS synthesis and of virus synthesis were very similar, which indicates that the retardation of the virus synthesis may be viewed as a direct result of

the retardation of the RNS synthesis. Accordingly, the pre-stage RNS indeed seems to get incorporated later into virus particles and is, therefore, not a "side product". It was possible to reinforce this view even further: When 5-fluor uracil was added only at the time of the near-maximum RNS titer, e.g., still just prior to the virus formation, it could no longer retard the formation.

When given full weight, these results seem to justify the conclusion that the virus RNS synthesis and the virus protein synthesis occur independently of each other. But unfortunately the work of Sanders is lacking in experimental detail and important data about the effect kinetic of the employed inhibitors; its evaluation is, therefore, not quite certain.

Wecker (90) found in the case of the WEE virus that tissue cultures infected with it yielded already after one hour an infectious RNS. The pre-stage RNS can here again be proved directly without difficulties, since under the same conditions the RNS incorporated in the virus is not released (43). The pre-stage RNS reproduces itself exponentially and reaches its maximum also at the commencement of virus reproduction. But contrary to examples mentioned earlier, the titer of the pre-stage RNS remains practically constant and does not decrease appreciably.

In order to examine the connections between RNS synthesis and protein synthesis, experiments were conducted with amino-acid analoga which can influence the protein synthesis. With Äthionin, the analogon of Methionin, the synthesis of the pre-stage RNS of WEE remained unaffected under all conditions, while infectious virus remained absent when the inhibitor was added prior to the virus reproduction, e.g., circa 4 hours p.i. or earlier. The retardation of the virus synthesis was irreversible. By counting virus particles under the electron microscope it was found that the number of the produced virus particles was almost identical with that of virus particles produced by control cultures not retarded. But the difference in the infectiousness of the synthesized viruses was indicated by the fact that the proportion infectious: total virus particles was more than hundred times greater in the case of Äthionin addition. These experiments were interpreted to mean that virus particles are indeed build up in the presence of Äthionin, but that they are not infectious, depsite the incorporation of infectious RNS, because they contain in their protein cover a false amino acid, namely Äthionin. This could have possibly changed sufficiently the normal surface characteristic of the virus particles (E. Wecker: Unpublished Results). The results were as follows with the amino acid analogon fluor phenylalanin (FPA):

When the inhibitor was added prior to the RNS synthesis the synthesis did not occur at all (90). After the optimum RNS titer had

been reached, the FPA was still able to retard the virus synthesis. In both cases was the inhibition completely reversible. Additional examinations indicated that most probably the retarding action of FPA on the RNS synthesis was not due to the fact, as in the case of bacteriophages, that virus nuclein acid is only synthesized when at first some new enzymes are provided for it. Instead Wecker found that no new enzymes are required for the synthesis of the WEE-RNS and that apparently the normal cellular apparatus was entirely sufficient. The result was that the retardation of an RNS synthesis with a known inhibitor of protein synthesis (93, 94, 95), namely FPA, probably must be construed to mean that RNS synthesis and protein synthesis directly depend upon another and concur timely. But whether this protein is the real virus protein remains entirely to be answered. But it can be said that the virus antigene, e.g., the virus protein, regularly is found far earlier in the cell than the first new complete virus (96). Here again is a parallel between pre-stage RNS and virus protein.

Corresponding direct examinations about the timely concurrence between the synthesis of nuclein acid and the appearance of intact virus in DNS-containing animal-pathogenic virus species are not yet available. But by means of more indirect techniques Salzman (97) and Magee et al. (98) were able to show that the synthesis of vaccine virus DNS also commences several hours prior to the appearance of ripe virus particle.

The phages DNS synthesized in infected bacteria was the subject of extensive examinations. But this field is already so broad that it was treated in special reviews and that its treatment here would go beyond the scope of the subject (99, 100).

## E. RNS AND PROTEIN SYNTHESIS

The designation of a virus RNS as "infectious" means that it not only transfers the necessary information for its own identical reproduction to the cell, but also for the synthesis of the corresponding specific virus proteins. And beyond that, the infection of cells with such virus RNS causes the same symptoms as they are observed after an infection with the complete virus. As mentioned above, a typical disease pattern comes into being in plant or animal after infection with virus RNS. This means that the nuclein acid component of a virus really can be the carrier of all those biological functions which are directly connected with the reproduction of the virus.

But infectious RNS has already been isolated in viruses of various size, e.g., in viruses possessing various absolute amounts of protein.

On the other hand, it is known and it was stated in the beginning that the absolute weight amount of RNS is practically the same in all examined RNS-containing virus species. This is once again presented in Table 9.

Table 9

| Virus Species | Particle Weight of Virus | Relative Content of RNS in % | Absolute RNS Amount per Virus |
|---|---|---|---|
| TMV | $40 \times 10^6$ | 5,6 | $2,2 \times 10^6$ |
| Polio | $6,7 \times 10^6$ | 22-30 | $1,5\text{-}2,0 \times 10^6$ |
| EEE + WEE | $50 \times 10^6$ | 4,4 | $2,2 \times 10^6$ |
| Classical Fowl Pest | $150 \times 10^6$ | 1,8 | $1,7 \times 10^6$ |
| Influenza | $280 \times 10^6$ | 0,7-1,0 | $2,0\text{-}2,8 \times 10^6$ |

From "Reproduction of RNA-containing animal vurises", W. Schäfer in: Virus growth and variation. 9. Symposium Soc. Gen. Microbiology.

But first it must be remembered that amounts of RNS per virus particle as calculated above agree very well with the molecular weight of infectious RNS determined directly in individual cases and obtained from these virus species (see front). Thus they contain probably only a single RNS molecule per virus particle. But how can an RNS molecule of almost or exactly the same size be able to inform at one time an equivalent of $38 \times 10^6$ total weight of virus protein, as in the case of TMV, and at another time such of only $4.7 \times 10^6$, as in the case of the poliomyelitis virus?

Before this question could be stated in such manner, it had been determined by means of chemical and physical methods that the TMV has an inner RNS thread which is wrapped in circa 2,200 identical protein subunits (101, 102). A surface structure similar in principle was also given for tomato bushy shunt virus, turnip yellow virus, and polio virus (103, 104). Thanks to improved techniques in presenting surface structures under the electron microscope (105) Horne and colleagues (106) was able to show that the "soluble antigen" of myxoviruses, e.g., their internal nucleoproteid, also has this principle of construction. It is very tempting to speculate that possibly the protein cover of all virus species is constructed of many identical protein units.

The answer to the question posed above would accordingly be that every virus RNS probably carries only the information for one kind of corresponding protein unit. A "large" virus would then possess many of such identical proteins, a "small" one correspondingly fewer.

The size of the protein subunit of TMV is known. It is a peptid of the molecular weight circa 1,800 and consists of 157 amino acids (107). Its RNS, in contrast, consists of 6,000 mononucleotids (45). Under the assumption that the virus RNS carries exclusively the information for the virus protein as such, it would require circa 40 nucleotids for each of its amino acids to fix them in their place within the peptid chain. This may seem to be a considerable waste of "information", since purely mathematically three mononucleotids per amino acid would suffice to order each in a specific sequence (108, 109, 110, 111).

Ycas (112) even proposes a "coding ratio" of 1, which then however would mean an additional genetical information of cellular material for the synthesis of an RNS virus.

But the order of magnitude of circa 40 nucleotids (40-120) corresponds to the one of a low-molecular RNS, to which is ascribed a specific transmitter role for amino acids during the protein synthesis (113).

This so-called S-RNS ("S" for soluble), or lately also "transfer RNS", seems to receive activated amino acids from an enzyme and then settle on the ribosomes within the endoplasmatic reticulum. In any case, it would be proved that the amino acids tied to S-RNS later are genuinely assimilated in peptids, a process which takes place on the ribosomes mentioned above. Of special importance is the finding that each amino acid has its specific activating enzyme and also a certain S-RNS meant only for it (113). Gierer (114) found in comparative examinations that a part of the RNS of ribosome has the same molecular weight, namely circa $2 \times 10^6$, as an infectious virus RNS. If now every RNS molecule with its specific amino acid attaches itself by means of hydrogen bridge on an equivalent distance of the high-molecular ribosome (or virus) RNS as it has been suggested (113), then the amino acids automatically would be brought into a sequence corresponding to the "information" within the high-molecular "template RNS". But in this case it would have to be demanded even in theory that the produced peptid consist only of circa 150 amino acids.

The above-described change effect between RNS and amino acids during the protein synthesis is up until now the only evidence of a possible mechanism for the protein synthesis which has been at least partly justified by experiments and which moreover would explain why, for instance, the protein subunit of TMV consists of only 157 amino acids.

But the stated hypothesis can be maintained only if really no other by the virus RNS "informed" proteins are synthesized in the cell infected with TMV. And until now, such have not been found (115). The occurrence of specific symptoms on the plant may therefore be construed,

for the time being, as a direct result of virus reproduction without new enzymes or other proteins playing a rule in it. As already mentioned in the preceding chapter, the participation of new enzymes in the synthesis of WEE pre-stage RNS has already been extensively discounted (90).

Finally it should be once more pointed out that for the infectiousness of a virus RNS the total integrity of the molecule is required, e.g., all of its circa 6,000 nucleotids. If only a part of it were responsible for the virus reproduction as such, and another part for the production of the symptoms, it could not be explained very easily.

Thus, with the present understanding the assumption is justified that a virus RNS of 2 million molecular weight is able to inform exclusively a protein with circa 157 amino acids. In view of this assumption, it becomes clear why reports about an infectious RNS from the viruses of the myxo group, influenza virus for example, must be received with great skepticism. These virus species are composed of two different substructures, each of which contains at least one specific protein. Myxo viruses, too, possess in all probability only one molecule RNS with a molecular weight of 2 million (see Table 8). It is therefore not merely coincidence that several work groups were unable to extract infectious RNS from these virus species in sufficient quantities and qualities to stimulate the formation of normal and intact viruses in a cell. All known and certain cases of infectious virus RNS derived from virus species which, as far as is known, possess only one type of specific protein.

The extremely close relations between virus RNS and virus protein synthesis are expressed again in experiments which are the subject of the following chapter, since they are of importance by themselves.

## F. THE IN-VITRO MUTATION OF VIRUS NUCLEIN ACIDS THROUGH CHEMICALS

If the nuclein acids in general can be considered the chemical equivalent for the phenomenon of inheritance, then mutations should be caused by corresponding chemical changes of this substance. Experiments to produce virus mutations deliberately through physical treatment of cells infected by virus, for example by X-rays, were successful (116, 117, 118, 119). Plants infected with TMV and kept at raised temperature already stimulated the production of numerous mutations (120). A more direct proof was submitted by Benzer and Freese (121) with their success in letting DNS of bacteriophages assimilate

unphysiological base homologes through the respiration of the bacteria. This led to a considerable yield of mutations in the progeny of such phages.

But the spontaneous, very small rate of mutation of viruses was unalterable by any of the listed methods when the viruses were exposed in vitro to such treatment.

Experiments with infectious virus RNS had shown that the RNS molecules of various virus species differ physically only insignificantly. Their specificity was therefore to be found in the special linear sequence in which the circa 6,000 nucleotids are connected in the macromolecule. Consequently, the RNS of a virus mutation was to differ then only in its base sequence from the one of the wild strain.

For the production of "mutated" virus RNS two conditions had therefore to be fulfilled:

1. The original sequence of the pyrimidine and purine in the RNS had to be changed appropriately;

2. The physical integrity of the RNS molecule had to be fully preserved in the process, since only it possesses biological activity, e.g., infectiousness.

Schuster and Schramm (122) showed that treatment of pyrimidines or purines with nitrous acid even at relative low $p_H$ led to oxydative desamination, e.g., the exchange of an amino group ($-NH_2$) for a hydroxyl group (-OH), regardless whether the bases were present in an RNS in free form or in form of nucleotids. Of the four normal bases of RNS of TMV three react as described above. Cytosin thereby becomes uracil, adenin becomes hypoxanthin, and guanin becomes xanthin. The fourth group, uracil, cannot react because it has no amino group. Interesting is especially the transformation of cytosin to uracil, since both are naturally existing bases of the virus RNS. In contrast, the reaction products hypoxanthin and xanthin are unphysiological for TMV.

Schuster and Schramm could furthermore prove that through the reaction of infectious RNS from TMV with nitrous acid under favorable conditions the physical structure of the RNS will not be changed. A quantitative evaluation of their experiments showed that through the chemical change of each one of at least 3,000 nucleotids, an inactivation of the RNS infectiousness is caused. But as is known, an infectious molecule consists of 6,000 nucleotids. In order of magnitude, a quarter of them, e.g., 1,500, contain the nonreacting base uracil. It was therefore possible that with nitrite also such nucleotids reacted whose change is not lethal. It possibly could then have been mutable.

This was first proved by Mundry and Gierer (124, 125) in the case of TMV. As genetical "marker" the authors used the variety of disease symptoms which appeared on a certain host plant (Java tobacco) after infection with wild strain TMV or a naturally present virus mutant. The "vulgare" strain caused a systematical illness of the plant which shows itself only in chlorotically discolored spots on the leaves. The mutant strain "dahlemense", however, caused distinct necrotical spots in the place of the infection, the number of which depends on the number of infected virus particles.

It was examined whether the treatment of infectious RNS from the strain "vulgare" with nitrous acid in vitro would lead to the production of Java necrose mutants. The spontaneous rate of mutation for this system is circa 0.2%. Through nitrite treatment it could be raised to 5.65%, e.g., at least twentyfold. This maximum increase of the mutation rate was observed when by concurrent reaction of "lethal" nucleotids the total infectiousness of the preparation was reduced by circa one half. This result alone excludes that this was not a production of mutants but only a selection of mutated viruses which had already existed before.

After prolonged reaction with nitrite was, if at all, only a very small fraction of the "surviving" RNS not mutated. The presence of protein did not disturb the in-vitro mutation. Intact TMV particles also reacted in basically the same manner to the treatment.

The quantitative evaluation of the results showed that the production of mutants through nitrite follows a one-hit-kinetic. The oxydative desamination of a single suitable nucleotid in the virus RNS may therefore be the occurrence of a mutation. The produced mutants are apparently stable and pass on their mutated character unchanged to their progeny.

As was to be expected, the treatment with nitrous acid did not always result in the same kind of mutation, for example, not always in a Java necrose strain. The above data about the increased rate of mutation, which refer only to this kind of change, do therefore not reflect the total of all mutations produced. The total should be considerably higher.

The principle of an in-vitro mutation through chemical change of the nuclein acid by nitrous acid, shown above by the example of TMV, should of course also be applicable to all other RNS-containing virus species. Surprisingly enough, so far only one other work has been published, reporting about the mutation of polio virus or its RNS by nitrite (126). The rate of a reverse mutation, reported therein, from the so-called d-character (attenuated strains) to the wild strain character $d^+$ (127) was remarkably low, namely, only circa three times

the rate of the spontaneous reverse mutation (128). It is improbable that these differences are based on general differences in physical and chemical structure between TMV and polio RNS. Firstly, both kinds of isolated virus RNS act similarly in many other respects; and secondly, the polio RNS is inactivated through nitrous acid with the same speed as the one of TMV. This means that the number of "lethal" groups able to react must be the same in both cases. If therefore the induced mutation rates are so different, then this could better be attributed to the fact that each RNS molecule "marker" carries different mutation probabilities. In other words, a polio RNS treated with nitrite may very well be mutated, even if this event is not expressed in form of a change of the d-character. But since the test system permitted only to prove these, all other mutants would have been overlooked. The reason for the obvious difficulty to produce in-vitro mutations in animal RNS viruses lies probably more in the limitation of our knowledge about suitable genetic markers than in the process of mutation production proper.

And indeed it was possible to produce in other, genetically better known systems in-vitro mutations with nitrous acid. In these cases DNS was the genetical material.

Vielmetter and Wieder (129) obtained with $T_2$ bacteriophages maximum 3% of a certain plaque mutant. Selective or other unspecific factors could be excluded. About similar findings reported Freese (130) with the T phage and Tessmann with the $\phi$ X 174 phage (131). The mutation of "transforming principle" was also successful with this method (132). In all cases inactivation and mutation followed a kinetic of the first order, which indicates that the change of a single nucleotid in the sense of a oxydative desamination suffices.

A two-hit-kinetic for the mutation of E. coli strain B through treatment of the bacteria with nitrite was observed by Kaudewitz (133). An optimum of 4% auxotrophic mutants was thereby produced.

Of greatest interest was the question: which of the three reacting organical bases would lead to a mutation event when desaminated. On the base of thorough kinetical studies Schuster (134) was able to prove that the desamination rate $\alpha$ depends on the H-ion concentration of the reaction mixture, and that adenin and cytosin act similarly, but guanin differently. Vielmetter and Schuster (135) then compared the desamination rate of the concerned pyrimidin or purin at various $p_H$ with the observed mutation rate of bacteriophages. It was found that the desamination rate for adenin and cytosin is equal to the mutation rate, while the value $\alpha$ for guanin distinctly differed from it. From this it can be concluded that the desamination of adenin or of both may constitute a mutation event, but not the one of guanin.

According to newer examinations by Tsugita and Fraenkel-Conrat (136), a mutation of TMV RNS can also be produced by methylation or bromation of their organic bases.

The listed results are apt to have far-reaching theoretical and practical consequences since with them an experimental possibility is given to mutate a genotype through direct and in principle understood chemical transformation of the genetical material. Furthermore the experiments prove again that each individual nucleotid of a virus nuclein acid is essential for the entire biological activity of the molecule, namely, for the infectiousness or the genetical information, or as it is expressed in a work by Siegel: "Each desamination (within the virus RNS) results in a provable biological effect" (137).

It is admittedly outside of present experimental possibilities to analyze the entire sequence of the circa 6,000 nucleotids of an infectious RNS and to determine thereby exactly what correlation exists between the chemical changes of one or of several of them and a certain genotype. But after Anderer and colleagues (107) have succeeded in compiling the entire amino acid sequence of the protein subunits of TMV, another highly interesting possibility appears: Does the amino acid sequence of protein subunits from the wild strain type change when its RNS has been chemically mutated? This question has already been experimentally approached by Wittmann (138). The tryptical peptids of various known TMV strains differ from each other slightly in grade of amino acid composition (139). But all possess the same total number of amino acids in the protein subunits, namely, 157 (140).

Wittmann compared now the amino acid composition of the 12 tryptical peptids from proteins of the wild strain "vulgare" and a nitrite mutant "Ni 54". The latter was produced through relatively long effect of nitrous acid on "vulgare" and subsequently singled out as mutant strain. There exists even the possibility that the RNS of this mutant differs in more than one nucleotid from the RNS of the vulgare strain.

If, as discussed in the preceding chapter, the entire virus RNS carries exclusively the information for the virus protein as such, and not for any other kind of protein, then every change in the nucleotid sequence should also express itself in a changed amino acid sequence. This would at the same time also give a good experimental indication of the theory that each individual amino acid of the virus proteins requires circa 40 nucleotids in the RNS in order to be correctly placed. In other words, the so-called "coding ratio" would be 40, and not three.

But Wittmann (139) found that neither the tryptical peptids nor their amino acid composition between "vulgare" and "Ni 54" were different. But it remains still to be examined whether the amino acid sequences are also identical, since after all the composition would

remain unchanged even if two certain amino acids changed places. But in principle this would still mean a different protein molecule.

In contrast to this, Tsugita and Fraenkel-Conrat (141) found in some of their mutant TMV strains, which also had been produced by chemical treatment in-vitro, that up to three amino acids in the protein subunits are different than in the initial strain. It is especially remarkable that two of these mutants are exactly the same amino acids, although the two strains, through the symptoms they caused on the host plant, biologically thus, differ markedly. In addition, one mutant strain was produced by methylation, the other by bromation (141). Different chemical changes in the bases of the virus RNS can therefore cause the same changes within the virus protein.

In the case of spontaneous mutation of TMV, it has also been known for some time that their protein cover in regard to amino acid composition (142, 143, 144), electrophoretical mobility (145), and antigenity (146) differs from the original virus strain.

The extremely important question of the coding ratio of virus RNS can therefore at the moment not be answered with certainty. At this time it looks as if some "in-vitro mutants" of TMV have different amino acid compositions, but others do not. Whatever the outcome, in these experiments again can be seen the intimate connection between RNS and protein, just as it has been pointed out by many other observations.

## G. CONCLUDING VIEWS

The definition of the viruses as "vagrant genes" as mentioned in the beginning has proved itself again in the light of newly gained knowledge. Indeed, this catchword describes completely the main characteristics of a virus!

The gene-character of a virus is on the base of experimental findings of the last few years, which culminated in the proof of infectious properties of virus nuclein acid, to be ascribed exclusively to this part of the chemical virus structure. "Vagrant" the nuclein acid becomes, because the protein specifically informed by it forms a cover, which protects this labile genetical material against the many environmental influences. But the protein cover is not required under favorable conditions, and the nuclein acid appears as the only really necessary virus structure, the only really infectious agent. For identical reproduction the virus nuclein acid requires an enzymatical apparatus as it exists in the living cell.

It is entirely possible that for the reproduction of virus nuclein acid and protein in the case of some more simple structured RNS viruses, the existing enzyme equipment of the cell is completely sufficient.

In contrast to this, it has been proved that for the reproduction of certain bacteriophages in the infected cell not fewer than 25 different proteins are synthesized, of which about two-thirds are completely new enzymes (99).

But it was the intention of this review to point out clearly the principles which always stand out in extreme cases. A simple ribonucleo protein which has all the characteristics of a "vagrant gene" without doubt must be termed an "extreme", as an extreme biological simplification. In addition to applied virus research, which deals mainly with virus species as the provocateurs of often serious and epidemical diseases, virology has therefore a theoretically much more far-reaching significance. On the model case "virus" knowledge has been gained which has furthered considerably our understanding of general biological phenomena. Into this group belong especially those, which gave detailed information about the significance of nuclein acids as the genetical material or about the relation between chemical structure and genetical information. Today it seems probable that some decisive problems dealing with the two most important substances of living matter, the nuclein acids and the proteins, are brought close to a solution through further studies of the relation between virus and nuclein acid.

H. TABULAR SUMMARIZATION

| Virus Species | Base Material | Extraction Method |
|---|---|---|
| | | Plant-Pathogenic |
| Tobacco Mosaic Virus | Cleaned Virus | Phenol + 4° C |
| " " " | " " | Dodecylsulphate |
| Tobacco ringspot virus | " " | 1 M-NaCl at 95° C |
| Tomato bushy stunt virus | " " | Dodecylsulphate and Phenol 6° C |
| Turnip yellow mosaic virus | " " | 1 M-NaCl bei 95° C |
| Necaotic ringspot virus | Infected Leaves | Phenol + 4° C |
| Tobacco rattle virus | Cleaned Virus | Phenol + 5° C |
| Potato x-Virus | " " | Denaturized Protein by means of Guanidin (see Reference 78) |
| Cucumber mosaic Virus | Infected Leaves | Phenol + 4° C present during homogenizing of leaves |

OF INFECTIOUS NUCLEIN ACIDS

| Test System | Peculiarities* | Reference |
|---|---|---|
| Virus Species | | |
| Leaves of nicotiana glutinosa | Infectiousness circa 2%, Molecular Weight of infectious RNS 2 Million, One-Strand Molecule, RNS | 30, 31, 33, 34 |
| " " " | Restitution with protein of TMV to Virus particle, infectiousness then approx. 70%, RNS | 29 |
| Leaves of vigna sinensis | Infectiousness 0.1-1.0%, RNS | 73 |
| Leaves of nicotiana blutinosa | Infectiousness 0.1%. Of the extraction methods, none yielded thus far infectious RNS singly | 74 |
| Leaves of brassica chinensis | Infectiousness 0.1-0.5%, RNS | 75 |
| Leaves of cucumis satirus | Infectiousness circa 1%, RNS | 76 |
| Leaves of phasereus vulgaris or nicotiana tabacum | Infectiousness circa 5%, RNS | 77 |
| Leaves of chenopodium amaranti color | Infectiousness 0.1-1.0%, RNS | 78 |
| Leaves of vigna sinensis | Infectiousness 50-500%. Infectious RNS probably derives not only from virus particle, but also from other intracellular material | 79 |

*All quoted percentage values represent the infectiousness of the isolated nuclein acid preparations relative to the virus infectiousness of the base material.

| Virus Species | Base Material | Extraction Method |
|---|---|---|
| | | Animal and Human-Pathogenic |
| Mengo-Virus | Infected Ehrlich-Ascites Tumor Cells | Phenol + 4°C |
| Encephalomyokarditis-Virus | Infected Ascites-Tumor Cells | Phenol + 4°C |
| Mice-Encephalomyelitis-Virus | Infected mice brain | Phenol + 4°C and + 50°C |
| | | Group |
| Eastern equine encephalo myelitis Virus | Infected mice brain or chicken embryos | Phenol + 4°C |
| Western equine encephalomyelitis Virus | Purified virus or infected tissue culture cells | Phenol + 4°C or + 50°C |
| Semliki forest Virus | Infected mice brains | Phenol + 4°C |
| | | Group |
| West-Nile-Virus | Infected Ascites Tumor Cells | Phenol + 4°C |

| Test System | Peculiarities | Reference |
|---|---|---|
| Virus Species Group Columbia SK | | |
| Mice i.c. Tissue Culture L-Cells, hypertonic | Infectiousness circa 0.1%. RNS derives from virus particles. Can be precipitated with 1M-NaCl. First animal-pathogenic RNS | 35 |
| Ascites Tumor Cells infected with RNS, then i.p. in mice, Plaque Test | Infectiousness circa 0.01%. RNS derives from pre-stage. Virus particles not extractable | 36 |
| Mice | RNS derived from pre-stage and from Virus particles. Cellular DNS has inhibitive effect. Belongs serologically obviously to Columbia SK Group | 37, 39 |
| Encephalitis A | | |
| Mice incubated chicken eggs, Plaque Test on chicken fibroblasts | Infectiousness circa 0.01%. RNS derived only from pre-stage. Virus inactivated by Athand, RNS not. Molec. weight of infectious RNS circa 2 Million | 41, 42 |
| Chicken egg | Infectiousness circa $10^{-5}$%. RNS from virus particles not extractable with cold phenol | 43, 90 |
| Mice | Infectiousness circa 0.1%. Virus inactivated by $D_1$ RNS not | 80 |
| Encephalitis B | | |
| Mice | Infectiousness circa 0.1%. RNS from Virus particle | 48 |

| Virus Species | Base Material | Extraction Method |
|---|---|---|
| Murray-Valley-Encephalitis-Virus | Infected Mice brain | Phenol + 4 C or 1% Desoxycholat |
| Tickborne-Encephalitis-Virus | " " | Phenol + 4 C |
| Dengue I and II | " " | Phenol + 4 C |
| Poliomyelitis-Virus Type I | Partially high-grade purified Virus | Entero<br>Phenol + 4 C and + 50 C, Dodecysulphate, cooking salt |
| Poliomyelitis-Virus Type II | Infected ZNS from Hamsters | Phenol + 4 C |
| Coxsackie A-7, B-4 and B-5 Virus | Virus-containing tissue culture medium | Phenol + 4 C |
| Coxsackie A-4 and B-1 Virus | " " " | Phenol + 4 C |
| Echo 1 and 8 | " " " | Phenol + 4 C |
| Theiler GD VII | Infected mice | Phenol + 4 C |

| Test System | Peculiarities | Reference |
|---|---|---|
| Fertilized chicken egg, mice | Infectiousness 1.0-0.1%. RNS from Virus. Heat-inactiv. Virus still yields infectious RNS | 46, 47 |
| Mice | Infectiousness $10^{-1}$-$10^{-3}$%. RNS | 81 |
| Young mice | Infectiousness 0.001-0.15%. RNS from Virus particles | 82 |
| Viruses | | |
| Plaque Test on H. and monkey kidney cells | Infectiousness circa 0.01%. RNS insensitive against DNase, Chymotrypsin and Papain | 49, 50 51, 52 |
| Mice | Infectiousness circa 0.1%. RNS derived from Virus particle | 48 |
| Plaque Test on H. and human A. cells | Infectiousness circa 0.1-0.01%. RNS derived from Virus particle | 83 |
| Tissue cultures of H., human A. and other cells | Infectiousness of RNS not certain: Test system cannot be infected by intact virus | 84 |
| Tissue cultures of H. and human A. cells | Infectiousness circa 0.1-0.01%. RNS derived from Virus particle | 83 |
| Mice | Infectiousness circa 0.1%. RNS derived from Virus particle | 82 |

| Virus Species | Base Material | Extraction Method |
|---|---|---|
| | | Other Small |
| Virus of the Foot and Mouth Disease | Infected mice | Phenol + 4°C |
| Virus of Maul and Klauenseuche | Infected mice brain, infected pork kidney-tissue cultures | Phenol + 4°C |
| | | Myxo- |
| Influenza A-Virus Asiatic Strain | Infected chicken membrane from chicken egg | Phenol + 4°C |
| Influenza A | Enriched virus | Ether pre-treatment followed by Phenol + 4° C |
| | | Tumor |
| Polyoma-Virus | Infected embryonic mice tissue culture | Phenol + 4°C, Phenol-Kirby-modification |
| [illegible] papilloma-Virus | Infected rabbit P. Warts | Phenol + 4°C<br>Phenol + 50°C |
| " " " | Partially purified Virus | Phenol + 4°C<br>Phenol + 50°C |
| Chloroleukemia-Virus | Leukemia tissue of infected mouse | Phenol + 4°C |

| Test System | Peculiarities | Reference |
|---|---|---|
| Virus Species | | |
| Young mice | Infectiousness circa 0.01%. Virus derived from Viruses and intracellular pre-stages Mol.-weight 3,1 Million | 53, 57 |
| Mice, Plaque Test on pork and cattle kidney cells | Infectiousness 0.01-0.001%. RNS derived from Virus and pre-stage | 54, 55, 56 |
| viruses | | |
| Tissue cultures of chicken kidney cells | Infectiousness uncertain, RNS-progeny of intect. cells not identical with original virus | 63 |
| Incubated chicken egg | RNS progeny serologically not identical with original virus | 60, 61 62 |
| Viruses | | |
| Tissue cultures from embryonic mice cells, infected. Medium of this tested in mice | Infectiousness not given. First infectious DNS. First infectious nuclein acid from a Tumor Virus | 67, 70 [illegible], für Kirby 69 |
| Rabbit skin | Infectiousness not certain: DNS may apparently derive from other material than Virus | 71 |
| " " | Infectiousness not certain: DNS derived from Virus particles | 72 |
| Newborn mice | Infectiousness not certain: First infectious RNS from Tumor Virus | 85 |

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